THE *IN VITRO* METABOLISM OF

C^{14}-LABELED PROGESTERONE IN BOVINE OVARIAN TISSUES

by

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INTRODUCTION

Long before any experimental evidence became available, there were many speculations regarding the function of the corpora lutea. Evidence that there was a secretory function of the corpus luteum was established by Corner and Allen in 1929 (7), who prepared the lipid extracts from corpora lutea and demonstrated that these extracts could maintain pregnancy in ovariectomized rabbits and stimulate development of an endometrium capable of maintaining an implanted fertilized ovum. The active extract of the corpora lutea was named "Progestin" which is employed to designate the crude or partially purified extracts. A pure form of progesterone was isolated in 1934 by several independent research groups at Rochester and Columbia Universities (10).

Early development of the progesterone assay measured the metabolite, pregnane-3α-20α-diol, in the urinary excretions. However, this method indicated there was no correlation between the urinary excretion of pregnanediol and the level of circulating progesterone. Further, pregnanediol may not be the only metabolite of progesterone; Δ^5-pregnene-3β-ol-20-one and cholesterol are also precursors of pregnanediol, and a number of related C-21 steroids were isolated from urine by Pearlman in 1948 (16).

In the last few years, more attention has been given to the quantitative determination of progesterone by chemical methods. Butt, Morris and Williams (6) developed a progesterone assay by polarographic estimation in 1951. Edgar in 1953 (9) reported a progesterone assay based on the partition between organic solvents and separated by the paper chromatography technique developed by Bush in 1952 (5). Gawienowski (12) in 1956 reported a modified method in the isolation and purification of progesterone by partition in various organic

solvents which was finally separated by chromatography on filter paper in a ligroin-propylene glycol system (20). The quantity of progesterone was measured by the ultra-violet absorption at 240 mμ in ethanol.

Since the determination of progesterone content in body fluid and corpora lutea has been extensively studied, a great deal of attention has been paid to the study of metabolic pathway of progesterone and its metabolites in the tissue and urine. It has been known since 1937 that the principal metabolite of progesterone was pregnanediol, which is formed by a reductive reaction at carbons 3,4,5 and 20 (8). The other metabolites, found in smaller quantities, are pregnane-3α-ol-20-one, allopregnane-3α-ol-20-one and allopregnane-3α,20α-diol. These were isolated from tissue, blood, and urine.

During the past few years, investigators of the metabolism of progesterone reported that in addition to the metabolites mentioned previously, some other intermediary metabolites have been isolated and identified. These investigations indicated that the metabolic pathway of C^{14}-labeled progesterone is linked to the C^{14}-labeled testosterone, and also that estrodiol was formed from the C^{14}-labeled testosterone.

In 1955, Baggett (2) reported that exogenously administered testosterone might serve as a precursor of estrogens in ovarian slice, and also Heard (14) reported in 1955, the same results with <u>in vivo</u> evidence. West and his co-workers (22) at the same time demonstrated that this conversion can occur in the human even in the absence of ovaries and adrenals. Therefore, these investigations provide a tentative link between the biosynthetic pathway of progesterone and estradiol.

This investigation was primarily the study of the <u>in vitro</u> metabolism of progesterone-4-C^{14} in bovine ovarian tissues, and it was undertaken in

order to better understand the metabolic role of progesterone in the ovary during the reproductive process. It was found that C^{14}-labeled progesterone was converted to C^{14}-labeled testosterone and C^{14}-labeled $\Delta^{1,4}$-androstadiene 3,17-dione in the cystic bovine ovarian tissue. The conversion of C^{14}-labeled progesterone to C^{14}-labeled testosterone has also been proved in the normal bovine ovary. This work was in confirmation of Solomon's (21) recent publication. Solomon reported that C^{14}-labeled progesterone was converted to C^{14}-labeled testosterone and C^{14}-labeled Δ^{4}-androstene-3,17-dione in the normal bovine ovarian tissue *in vitro*. The conversion of progesterone to testosterone that occured in the cystic bovine ovary was the first that has been demonstrated in the literature.

Continuing the work done by Gawienowski in 1956, the isolation of progesterone from bovine corpora lutea during pregnancy at the third stage was undertaken. Five additional steroid hormones were isolated from bovine ovarian tissue, corpus luteum and cystic ovarian follicular fluid, and have been tentatively identified. Further investigation of those steroids would be highly desirable, but the limitation of sources and the extremely low quantities isolated led to difficulties which prevented a positive identification.

The experimental methods of Edgar and Gawienowski for the isolation and identification of progesterone and related steroid hormones were followed. Samuels' (19) method was used for the study of the metabolism of C^{14}-labeled progesterone, and Savard's (20) paper chromatographic techniques were followed.

EXPERIMENTAL PROCEDURE

Chemical Methods Used in the Study of the In Vitro Metabolism of Non-Labeled Progestorene and Progesterone-4-C^{14} in Bovine Ovarian Tissues

The majority of the procedures followed in this investigation were taken from the Samuels' work (19). His group demonstrated that the rat testis can remove the side chain from progesterone in vitro and form testosterone and Δ^4-androstene-3,17-dione. A modification of his experimental work was made in this laboratory on the temperature of incubation and the paper chromatographic techniques used in the separation and identification of isolated steroid hormones.

Incubation. The frozen bovine ovarian tissues were sliced and homogenized with known amount of the bovine serum-buffer mixture which was made by mixing an equal volume of bovine serum and phosphate buffer at pH 7.4 which contained the following concentrations of co-factors: 0.04 mole nicotinamide, 0.35 millimole diphosphopyridine nucleotide, 0.31 millimole adenosine triphosphate, and 0.4 millimole fumaric acid. Two grams of the tissue homogenates were transferred into a 125 ml. glass-stoppered Erlenmeyer flask and washed with two portions of two ml. of the serum-buffer mixture. The latter was made to a total volume of 25 ml. for the incubation. One half or one micro mole of the C^{14}-labeled progesterone, (23,938,325 counts per minute per micro mole) purchased from Nuclear Chicago, was dissolved into 0.2 ml. of propylene glycol and added to the incubation flask. Incubation was carried out under a gas phase of 95 per cent oxygen and 5 per cent CO_2 for three hours with constant shaking in a water bath at 37°C.

Extraction. The lipid fraction of the incubation mixture was extracted by ethyl acetate four times (v/v) from the serum-buffer mixture. Each

extraction was performed by stirring the incubation mixture with ethyl acetate for five minutes and centrifuging at 2500 to 3000 rpm for twenty minutes. The supernatant, ethyl acetate extract, was decanted and the remaining portion which contained the serum buffer and tissue residue was discarded.

The combined extracts were washed with a saturated sodium bicarbonate solution to remove the estrogens, and then washed with double distilled water to remove the excess sodium bicarbonate solution. The washed ethyl acetate extracted was evaportated to dryness in vacuo.

Chromatography. The lipid portion of the extract was partially removed by the chromatography on a column of 110 mm. long and 11 mm. in diameter, into which was filled with four grams of silica gel (Davidson Chemical Corporation, 80-200 mesh.). The dried extract was dissolved in 10 ml. of a 1 : 4 (v/v), solution of ethyl acetate and hexane, and passed through the column. The first eluent containing most of the fat portion was discarded, since this fraction was shown to have no significant activity. The steroids absorbed on the column were eluted by 10 ml. of a 1 : 1 (v/v) solution of ethyl acetate and absolute methanol. The eluent was then evaporated to dryness under vacuo.

The partially defatted extract was chromatographed by the Savard technique (20) in the ligroin-propylene glydol system or the toluene-propylene glycol system on filter paper strips as mentioned in the previous method for progesterone assay.

Detection of Steroids on the Paper Chromatograms. The extracted steroid hormones were detected on the paper strips by a preliminary examination with an ultraviolet light scanner. The spots were marked by pencil in order to

outline the approximate area.

A chromatogram scanner was also used to detect the radioactivity and location of the unknown steroids. The paper strip containing the radioactive spots was scanned by a Geiger tube within the scanner, the activity (counts per minute) and the location of the active peaks were recorded by a count rate meter and registered on a recording sheet to give the exact location of the isolated unknown steroid on the chromatogram.

A Zimmerman color reaction was used to detect the hormone standards on the adjoining strips and to locate the unknown steroids in certain cases as an aid for identification.

Identification. R_T values, melting point determinations, and infra-red spectra of the isolated metabolites were the methods for identification. The R_T value is characteristic of each different steroid in a given solvent system used in paper chromatography, and is defined as the following:

$$R_T = \frac{\text{movement of unknown steroid}}{\text{movement of standard steroid}} \times \begin{array}{c}\text{rate of movement}\\ \text{of standard}\end{array}$$

$$= \frac{Du}{Dst} \times K_{st}$$

where D_u = the distance traversed by the steroid of unknown mobility

D_{st} = the distance traversed concurrently by the standard

K_{st} = the rate of movement of the standard in cm. per hour in the same solvent system at room temperature.

The isolated metabolite which had a R_T value corresponding to the hormone standard was eluted by 95 per cent ethanol from the paper strip and mixed with 10 mg. of the standard hormone as the carrier and recrystallized in ligroin, from which crystals were obtained for a melting point determination

on a Kofler micro hot stage. The unknown was also dissolved in benzene for placement on planchets for the determination of its radioactivity by a windowless gas flow proportional counter. The radioactive substance after several recrystallizations when mixed with the standard hormone as carrier had a constant melting point range corresponding to the standard hormone. It also had a constant specific activity which indicated a pure hormone. The infra-red spectrum of the compound and the hormone carrier showed characteristic absorption peaks identical to the spectrum of the standard hormone.

Chemical Methods in the Determination of Progesterone or Related Steroid Hormones From Corpora Lutea; Cystic Ovarian Tissues and Abnormal Follicular Fluid.

The procedure used in this investigation for the chemical determination of progesterone and related steroid hormones followed were taken from Edgar and Gawienowski's original methods comprising the preliminary extraction from the tissues and fluid; partition between organic solvents; separation by paper chromatography; detection on the paper chromatogram by ultra-violet light scanning and color reaction; and the qualitative and quantitative microanalysis by spectroscopy.

Extraction Method. The bovine ovarian tissue or corpus luteum was initially homogenized with 20 ml. of 95 per cent ethanol in a Warning Blender which was kept in a deep freeze for fifteen minutes previous to the homogenization. The tissues were then extracted for one hour in a Soxhlet extractor according to the method of Allen (1). This alcoholic extract containing the total lipid fraction from the tissue was then reduced to approximately 10 ml. by evaporation under vacuo. The condensed extract was diluted with 20 ml. of double-distilled water.

The diluted alcoholic extract was added with stirring to 100 ml. of a 3 : 1 (v/v) ethanol-ethyl ether mixture. The mixture was stirred for five minutes, centrifuged, after which the supernatant liquid was decanted. The precipitate was washed twice by stirring with 50 ml. portions of the 3 : 1 ethanol-ethyl ether mixture and then separated by centrifuging. The precipitate, composed of phospholipids, was discarded. The combined extract and washings were concentrated under vacuo to about 20 ml. and then diluted with 40 ml. of double-distilled water.

The diluted solution was extracted with three 60 ml. portions of ethyl acetate and the combined extracts evaporated to dryness under vacuo. The remaining alcoholic solution contained estrogens and was discarded. The residue was transferred to a centrifuge tube by washing the flask three times with 3 ml. portions of hot 70 per cent aqueous methanol. The methanol mixture was stored for eighteen hours at -15°C. and then centrifuged at -10°C. for ten minutes. The precipitate, composed of neutral fat and cholesterol, was discarded.

After centrifuging, the supernatant liquid was decanted, diluted with 20 ml. of double-distilled water and extracted with three 30 ml. portions of light petroleum ether (b. p. 40-60° C.). This will enhance the separation of progesterone from the remaining cholesterol which remains in the 70 per cent methanol was discarded. The combined petroleum ether extracts were washed with two 30 ml. portions of water and reduced to about 1 ml. under vacuo, then transferred with two washings of 0.5 ml. portions of light petroleum to a glass evaporating dish for evaporation to dryness in vacuo over $CaCl_2$.

Paper Chromatography. The filter paper sheets (Whatman No. 1, 46.5 x 10 cm.) were washed thoroughly in a Soxhlet extraction apparatus with hot methyl alcohol for a total of seventy-two hours in order to remove the impurities from the paper. These impurities are responsible for the interference in the spectrophotometric determinations.

The ligroin-propylene glycol system as described by Savard was used for the separation and identification of progesterone and other steroids. The washed filter paper sheets were completely dried and were cut into 1 x 45 cm. strips attached at the upper end to a common base which is about eight cm. in length. The paper chromatogram strips were impregnated with the stationary solvent (propylene-glycol) by dipping into a freshly prepared 50 per cent methanol solution (v/v) and removing the excess by blotting it between two sheets of filter paper. The blotted strips were left between the sheets of the blotting paper with only the upper end and starting lines projecting during the following operation. The crude extract dried over $CaCl_2$ was dissolved in benzene and applied to the strips in small volumes with the aid of a current of air. Known steroid hormones were applied to the adjacent strips for the subsequent identification of the relative mobility (RT) of the unknown steroids. The paper strips were hung in a cylindrical chamber (12'' x 24''), by placing the common base of the upper end of the paper strips into a solvent trough. The chamber was covered with a glass plate and sealed with clay, and allowed to equilibrate overnight with the mobile solvent (ligroin). The saturated paper strips were developed by adding 60 ml. of ligroin (b. p. 60-90°C) to the trough through a hole on the top of a glass plate. After the necessary period of time for chromatography, which varied according to the different mobility of the individual steroids being studied, the paper strips were removed from the chamber and suspended in a current of air and allowed to dry.

Detection of Steroids From Chromatogram. The steroids on the chromatogram were located by means of a modified Zimmerman color reaction and the ultraviolet scanning. The color reaction was proceeded by immersing the paper strip in a 2.5N solution of Potassium hydroxide in ethyl alcohol (freshly

prepared), removing the excess reagent by thorough blotting; dipping the strip in a 2 per cent solution of m-dinitrobenzene in ethyl alcohol, and again blotting the excess reagent. The colors were quickly developed by gentle warming of the strip. A blue-violet color was developed by 3-ketosteroids and a violet color by the 3-keto-C-19 steroids. This aided in the identification of the unknowns.

By scanning the chromatograms with ultraviolet light one can detect the alpha, beta unsaturated ketones at levels of 5 mcg. or less. The ketosteroids with an α, β - unsaturated to the carbonyl group in ring A can be localized visually on the chromatogram by the ultra-violet light, marked off and eluted with a 95 per cent ethanol solution for the further spectrophotometric determination.

Qualitative and Quantitative Microanalysis. The qualitative analysis of progesterone was performed as described previously by using the modified Zimmerman color reaction. This gives a distinct blue violet color at the area of the extracted hormone, as well as on the parallel strips which contained the progesterone standards. The exact quantity of progesterone isolated can be determined by the intensity of the absorption at 240 $m\mu$ in an ethanol solution. A reference blank solution was made by eluting a blank of filter paper at the area corresponding to the unknown extract on the same chromatogram. The ultra-violet absorption spectrum of the extract in concentrated sulfuric acid gave a specific absorption spectrum which was identical to the spectrum of progeserone.

The steroid hormones isolated, besides progesterone, were identified qualitatively by the Zimmerman color reaction, relative mobility (R_T value) of unknown compared to the standard hormones, and by the ultra-violet absorption spectra in concentrated sulfuric acid. A reference blank was used to compensate for the interference caused by the paper impurities.

RESULTS

Tentatively Identified Metabolites of Progesterone From Normal and Cystic Bovine Ovaries

In vitro metabolic studies of non-radioactive progesterone in normal and cystic ovaries were carried out in several experiments.

Experiment 1. In this investigation, a polycystic ovary (prevulatory stage) weighing 16.05 g. was used. The histological study of this ovary was indicated in Plate I on page 13. Two grams of the tissue were incubated with one micro mole of progesterone in 25 ml. of a bovine-serum mixture at 34°C for three hours.

Two samples were run at the same time. In sample No. 1 no metabolites were isolated, however, a steroid identified by a R_T value; Zimmerman color reaction; and infra-red spectrum was found to be progesterone.

From sample No. 2, a steroid was isolated which had a R_T of 2.9 and an intense blue color after development by the Zimmerman color reaction. The ultra-violet light absorption spectrum in concentrated sulfuric acid tentatively identified it as Δ^4-androstene-17 β -ol-3,11-dione.

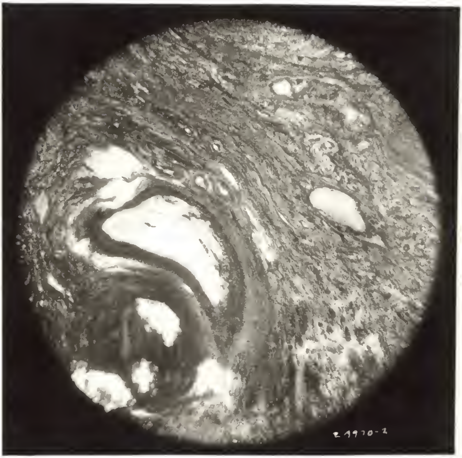
Experiment 2. In this experiment the same cystic ovarian tissue was used as in the first incubation. Progesterone was the only steroid isolated.

Experiment 3. In this experiment both normal and cystic ovarian tissues were used. Two normal ovaries (weighing 8 g. total) were taken from nonpregnant cows and incubated with eight mg. of progesterone (approximately 25.4 micro moles) at 37°C for three hours. One metabolite was isolated and identified as Δ^4-androstene-3,17-dione by the Zimmerman color reaction, R_T and the infra-red spectrum. Ten grams of a cystic ovary (34.4 g. total weight) were incubated with 10 mg. of progesterone (approximately 31.8 micro moles) under the same

EXPLANATION OF PLATE I

Cystic Ovarian Tissue (X 100). Polycysts developed among the fibrous tissue. Large cavity (a single cyst), near the center was surrounded by a narrow dark layer of the cells of Theca folliculi.

PLATE I



conditions. One metabolite isolated from the cystic ovarian tissue, which had a R_T of 2.5, a dark brown color from the Zimmerman color reaction as well as a characteristic ultra-violet absorption spectrum in concentrated sulfuric acid, was tentatively identified as Δ^7-allopregnene-3,20-dione.

Metabolites of Progesterone -4-C^{14} Isolated from Bovine Cystic and Normal Ovaries

In this investigation, two cystic and two normal ovaries were studied.

Experiment 1. A poly cystic ovary (88.9 g) was used in this incubation. A section of this ovary can be seen in Plates II and III on pages 16 and 18. A small sample of the tissues (2. g.) was incubated with 1 micro mole of progesterone-4-C^{14} in a water bath at 37°C for three hours. Two metabolites were then isolated by paper chromatographic techniques in a ligroin-propylene glycol system.

One metabolite located in the area corresponding to the standard testosterone spot on an adjoining strip showed a distinct dark spot when scanned with ultra-violet light. The other metabolite was in the area of Δ^4-androstene-3,17-dione. The paper strip containing the metabolites was cut into one inch sections from the starting line and counted with a windowless gas flow proportional counter. Two pronounced radioactive peaks were detected, one in the area of testosterone and other in the area of Δ^4-androstene-3,17-dione. The metabolite located at the area of testosterone had the same mobility as standard testosterone after being eluted and chromatographed a second time. The active area was eluted with ethanol into a 5 ml. volumetric flask, and the activity was counted by a gas flow proportional counter. The activity of this compound was 244,000 counts per minute (cpm), which indicated a 1.08%

EXPLANATION OF PLATE II

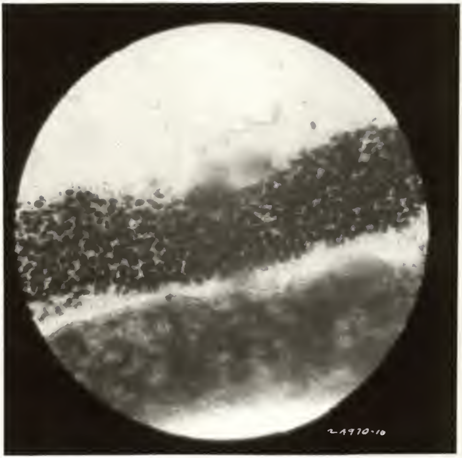
Cystic Ovarian Tissue (X 100). A large single cyst in the center position, which was comprised of follicular cells and Theca folliculi (shown by a dark narrow band). In the center of cavity, the floating follicular cells were shown by the dark colored bands.

PLATE II

EXPLANATION OF PLATE III

Magnified Follicular Cells and Theca Folliculi of Plate II (X 430). The dark band in the center of the plate indicated a layer of follicular cells which contained the round nucleus. The light band in between two dark bands was the inner, vascular layer of the Theca folliculi. The dark band at the outer edge of the plate was an external layer of the Theca folliculi.

PLATE III



conversion from 1 micro mole of progesterone $-4-C^{14}$ used for this incubation.

The active compound was mixed with 10 mg. of non-labeled testosterone and recrystallized in ligroin (b. p. 65-90°C) at room temperature. The crystals had a melting point of 151-154°C, which compared very well with the melting point of standard testosterone 151c-156°C (15), after the first recrystallization and a specific activity of 56,880 cpm per mg, was found. A melting point of 151-154°C and a specific activity of 56,580 cpm per mg. were determined after the second recrystallization. It indicated that the C^{14}-labeled testosterone isolated from the cystic ovarian tissue was in a pure form. After the removal of the crystalline testosterone, the solvent containing the recrystallized testosterone revealed no significant radioactivity.

The infra-red spectrum of the testosterone-C^{14} with carrier was identical to the absorption spectrum of the standard testosterone as indicated by Figs. I and II.

The other metabolite isolated in the area of Δ^4-androstene-3,17-dione was rechromatographed in order to obtain more information for further identification. It was found by a second chromatographic run that this active compound was not Δ^4-androstene-3,17-dione (which has a R_T of 1.7). The isolated compound had a R_T of 0.67 which was similar to the $\Delta^{1,4}$-androstadiene-3,17-dione which has a R_T value of 0.62 according to Savard (20) in the ligroin-propylene-glycol system. The unknown compound was eluted and rechromatographed in ligroin-propylene glycol system with 150 micro-grams of the standard $\Delta^{1,4}$-androstadiene-3,17-dione as the carrier. The unknown and the standard hormone traversed with the same mobility on the chromatogram. A dark spot was located by the scanning of ultra-violet light and a pronounced radioactive peak in the area of this dark spot was indicated by a paper chromatogram scanner. This active substance and the carrier were eluted by ethanol and counted by the gas

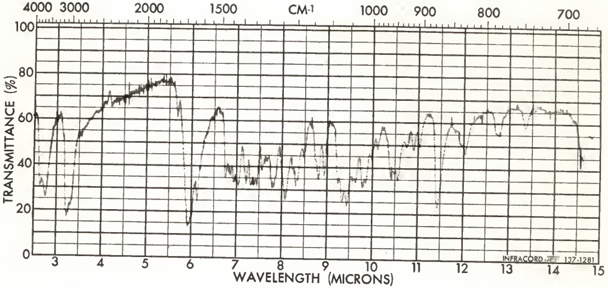


Fig. I Testosterone (Standard)

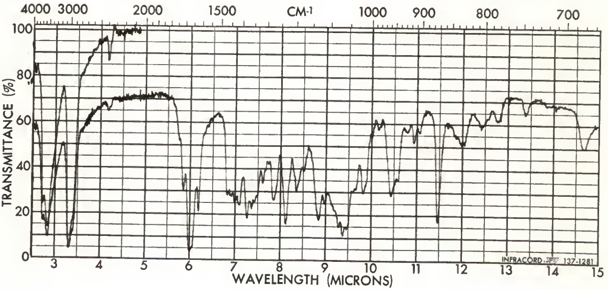


Fig. II Testosterone-4-C^{14} with the Carrier

flow proportional counter. A total activity of 5,070 cpm of this compound indicated a 0.0142 per cent conversion from 1 micro mole of progesterone-4-C^{14} incubated.

For further identification, the infra-red spectrophotometric determination of this compound was undertaken by using a micro potassium bromide pellet ($1\frac{1}{2}$ mm. in diameter) made by the lyophilization technique (17). The infra-red spectrum was determined by means of a beam condensing unit which was mounted on a model 137 Infracord. (Perkin-Elmer Corporation). Partly due to the scattering of the sample particles within the pellet, a very clear and distinct spectrum was not obtained. However, the various chacteristic absorption peaks were identical to the spectrum of the standard $\Delta^{1,4}$-androstadiene-3,17-dione. The infra-red spectra of the standard and the isolated $\Delta^{1,4}$-androstadiene,3,17-dione are given in Figs. III and IV.

The second cystic ovary (101.05 g.) was used for the following incubation. About 2 grams of tissue were incubated with 1 micro mole of progesterone-4-C^{14} under the same conditions as the first incubation.

In this incubation only the C^{14}-labeled testosterone was isolated. It was detected initially by scanning the paper chromatogram with ultra-violet light. A slightly dark spot was located at the area corresponding to the standard testosterone on an adjacent strip. By using a paper chromatogram scanner a pronounced radioactive peak was recorded at the area of this dark spot. The active testosterone was eluted with ethanol from the paper and the activity counted with a gas flow proportional counter. The total activity of the compound was 935,000 cpm, or equivalent to 1.78 per cent of incubation progesterone-4-C^{14} (1 micro mole).

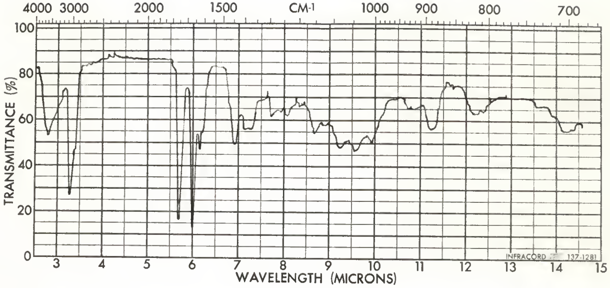


Fig. III $\Delta^{1,4}$-Androstadiene-3,17-dione (Standard)

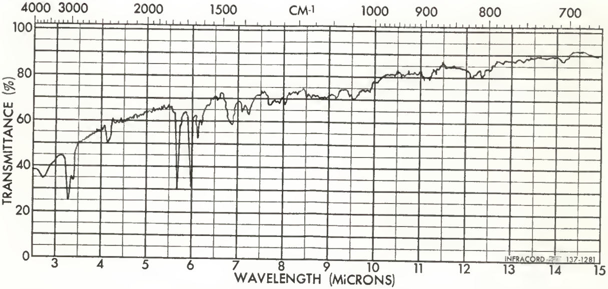


Fig. IV $\Delta^{1,4}$-Androstadiene-3,17-dione-4-C^{14} with the Carrier (150 mcg.)

Experiment 2. Two normal bovine ovaries were studied in this experiment. One normal ovary weighing 6.6 grams was used in the first incubation. A photomicrograph of this ovary was placed on Plate IV.

Two grams of the tissue were incubated with 0.5 micro mole of progesterone-4-C^{14} in a water bath at 37°C for three hours with constant shaking. The detailed procedures followed were identical to the previous experiments.

One metabolite was isolated from the incubation extract and found to have a mobility the same as the standard testosterone on an adjacent paper strip. Scanning by an ultra-violet light paper chromatogram scanner, a very high peak was located at the spot area corresponding to testosterone. The ethanolic eluent of this active spot was counted for its activity on a gas flow proportional counter and shown to have a total activity of 2,420,000 cpm. This was a 7.07 per cent conversion from the 0.5 micro mole progesterone-4-C^{14} incubated.

The eluent was evaporated to dryness and recrystallized with 10 mg. of the non-labeled testosterone in ligroin at room temperature. The crystalline testosterone obtained from the first recrystallization had a melting point of 153-155°C and a specific activity of 115,560 cpm. per mg. The melting point determined after the second recrystallization in ligroin was also 153-155°C, and the steroid had a specific activity of 116,446 cpm per mg.

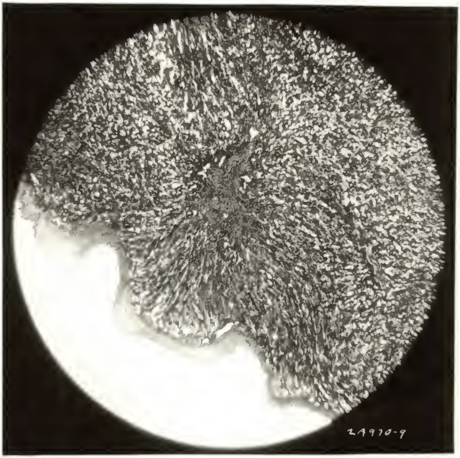
A normal ovary, (10.4 g.), containing a growing follicle, of non-pregnant cow was studied in the second incubation. Two grams of tissue were incubated with 1 micro mole of progesterone-4-C^{14} in a water bath at 37°C for three hours with constant shaking.

A metabolite was isolated after a second paper chromatographic separation in the ligroin-propylene glycol system. This compound had a R_T value identical to the standard testosterone which was on an adjoining reference strip. The

EXPLANATION OF PLATE IV

Normal Ovarian Tissue (X 100). Essentially fibrous tissue containing a degenerated corpus luteum scar, which indicated it was an ovary from an old cow.

PLATE IV



scanning by ultra-violet light on the paper strip showed an intense dark spot of this isolated compound. The eluent of this active compound was counted by a gas flow proportional counter and found to have a total activity of 726,500 cpm. The percentage of conversion from progesterone-4-C^{14} was calculated as 2.28 per cent.

The Quantitative Analysis of Progesterone From Bovine Corpora Lutea During the Third Stage of Pregnancy

The corpora lutea of cows killed during the third stage of gestation (approximately 90-129 days) were analyzed for progesterone. The quantity of progesterone isolated varied with individual corpus luteum. An average of 5.2 mcg. of progesterone per g. of tissue was obtained from the analyses of the four corpora lutea. Amount of progesterone isolated from each sample is shown in table 1.

Table 1. Progesterone Content of Bovine Corpus Luteum.

Sample No.	Weight of Sample g.	Weight of Progesterone mcg./sample	Progesterone Content mcg./g. of sample
1	5.35	26.2	4.4
2	6.10	16.8	2.8
3	6.05	19.8	3.2
4	4.45	46.5	10.4
Average	5.49	27.3	5.2

The progesterone was isolated on the paper chromatogram by scanning the paper strip with ultra-violet light. This procedure reveals the compound as a dark spot against the purple flourescent background of the paper. The spot corresponding to the standard progesterone spot on an adjoining strip developed

into a blue-violet color by the Zimmerman color reaction. This was the same as the color for progesterone. The R_T value of the unknown spot was 2.3 which was identical to the R_T for progesterone.

The hormone was normally eluted from the paper strip with ethanol into a 5 ml. volumetric flask in thirty minutes. It was then measured in a Beckman D. U. Spectrophotometer from 220 $m\mu$ to 320 $m\mu$. against a reference blank solution which was eluted from an adjoining blank strip to compensate for the interference caused by the paper "dirt". The absorption spectrum of the isolated hormone showed a charateristic maximum absorption at 240 $m\mu$, which was identical to the standard progesterone.

The quantitative determination of progesterone was calculated on the basis of Beer's Law with known concentrations of progesterone. The absorptivity of progesterone is a linear function of the concentration of the steroid within the range of 40 to 50 mcg. per ml. and obeys Beer's Law.

The Quantitative Analysis of Progesterone From Bovine Cystic Ovarian Tissue and Cystic Ovarian Follicular Fluid

The cystic ovarian tissue and the follicular fluid, supplied by the Dairy Husbandary Department, were analyzed for progesterone (Table 2).

Table 2. Progesterone Content of Cystic Bovine Ovarian Tissue and Fluid

Sample	Weight of Volume of Sample	Weight of Progesterone mcg.	Progesterone content mcg. per unit weight or volume
Tissue	36.8 g.	10.0	0.3 mcg. / g.
Follicular fluid	17 ml.	143.0	8.4 mcg. / ml.
Follicular fluid	1 ml.	1.2	1.2 mcg. / ml.

The quantitative and qualitative analysis of progesterone were made in the same way as demonstrated for the analysis of progesterone from corpora lutea.

Recovery of Progesterone Added to the Organic Solvent

In order to know the accuracy of the methods used for progesterone analysis, a study of the recoverage of progesterone was undertaken. A quantity of 50 mcg. of progesterone in ethanol solution was added to 100 ml. of ethanol-ethyl ether mixture 3 : 1 (v/v) and assayed by the same procedures as used in progesterone analysis. A 96 per cent recovery of progesterone was obtained in this experiment.

Steroids Isolated From the Bovine Corpus Luteum and Cystic Ovarian Follicular Fluid

In the determination of progesterone from a corpus luteum (sample No. 2), another steroid was isolated, which had a R_T value of 3.2 in the propylene-glycol system after being developed nine hours at room temperature. The characteristic absorption spectrum of this compound in concentrated sulfuric acid measured from the region of 220 $m\mu$ to 300 $m\mu$ showed it to be pregnane-3,20-dione.

In the cystic ovarian follicular fluid, a hormone was isolated and tentatively identified to be androstane-17β-ol. This steroid gave a green color of fluorescence when exposed to ultra-violet light and a R_T value of 3.5 in the propylene-glycol system. Ultra-violet light absorption from 220 to 330 $m\mu$ in concentrated sulfuric acid gave a characteristic absorption spectrum identical to the androstane-17β-ol.

DISCUSSION

The results from the quantitative analysis of progesterone of the cystic ovarian follicular fluid showed that the quantity of progesterone isolated from one sample was greater than the other. Owing to difficulties in getting more samples, a limited number of analyses were made. However, this proves the availability of progesterone for biosynthesis to testosterone in the ovary. The methods used for the quantitative determination of progesterone as modified by Gawienowski (12), (13) was very precise and sensitive at the low levels of progesterone found within the tissue or fluid.

Tentatively identified metabolites of non-labeled progesterone from the normal and cystic ovary <u>in vitro</u> gave an indication that the metabolism of progesterone in the cystic ovary might follow a different metabolic pathway from the normal ovary. Δ^4-androstene-3,17-dione for instance has been tentatively identified in the normal ovary. Solomon (21) has shown that progesterone was converted to the androgens. The tentatively identified metabolites from the cystic ovaries were Δ^7-allopregnene-3,20-dione. Therefore, it is postulated that progesterone might followed the different metabolic pathways in the cystic ovaries.

The study of the metabolic pathway of progesterone-4-C^{14} in cystic and normal bovine ovaries were carried out successfully in this preliminary investigation. It seems that the ovarian tissue contained an enzyme system which could remove the sidechian of progesterone to form C-19 steroids. The conversion of progesterone-4-C^{14} to the C^{14} labeled testosterone in the cystic and normal ovaries was well established. The formation of $\Delta^{1,4}$-anrostadiene-3,17-dione in the cystic ovarian tissue also was proven. It was postulated that the

metabolic pathway of progesterone-4-C^{14} in the cystic ovary was via $\Delta^{1,4}$-androstadiene-3,17-dione; Δ^{4}-androstene-3,17-dione and to testosterone. Another possible route via 17α-hydroxy-progesterone; $\Delta^{1,4}$-androstadiene-3,17-dione; Δ^{4}-androstene-3,17-dione and to testosterone as indicated in Fig. 5. Although the formation of 17α-hydroxy-progesterone from progesterone was not discovered in this investigation, however, the formation of 17α-hydroxyprogesterone in the bovine normal ovary as reported by Solomon (21) was well established.

A point worth noting was that the quantity of C^{14}-labeled testosterone converted from progesterone-4-C^{14} was greater in the normal ovaries than in the cystic ovaries. These preliminary results were consistent with the investigations reported by Garm (11) in 1949. He demonstrated that the urinary excertion of the neutral steroids were constantly lower in nymphomanic cows than in normal cows. The low yield of testosterone in the cystic ovaries might be due to the enzymic block. However, the mechanism of this conversion remains for further investigation. The percentage of C^{14}-labeled testosterone formed from the progesterone 4-C^{14} is indicated in table 3.

Table 3 Percentage of C^{14}-Labeled Testosterone Formed From C^{14}-Labeled Progesterone in Normal and Cystic Bovine Ovaries

Sample Number	Weight of Sample	Amount of Progesterone Incubated	Amount of Testosterone Formed	Percent
1. Normal Ovary	2g.	0.5 micro mole	0.03534 micro mole	7.07
2. Normal Ovary	2g.	1.0 micro mole	0.02280 micro mole	2.28
3. Cystic Ovary	2g.	1.0 micro mole	0.01080 micro mole	1.08
4. Cystic Ovary	2g.	1.0 micro mole	0.01780 micro mole	1.78

The histology of the normal and cystic ovarian tissues used in these investigations have been studied. The photomicrographs of these ovarian tissues are shown in Plates I, II, III, and IV.

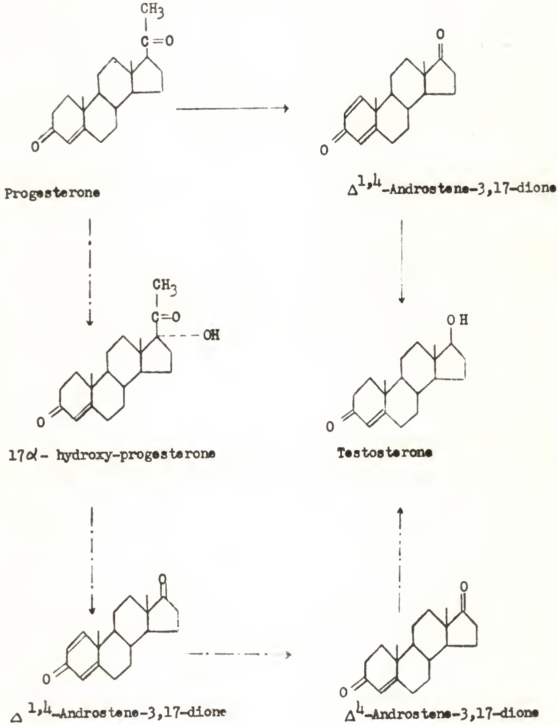


Fig. 5. Two possible metabolic pathways for progesterone in the cystic bovine ovary.

A remarkable difference between the normal ovarian tissue and the cystic's was the number of the cavities (cysts) which contained follicular fluid in the cystic ovarian tissue. These cysts are believed to be responsible for the essential symptoms of nymphomania in the cow.

The results on the quantitative analysis of progesterone from bovine corpora lutea during the third stage of pregnancy, indicated that there was no correlation between the weight of tissue and the quantity of progesterone isolated. The average amount of progesterone isolated from four individual corpus luteum was 5.2 mcg. per g. of tissue. This result was consistent with the results obtained by Rakes, et al, in 1958 (19), they reported that an average amount of progesterone determined from the corpora lutea of thirteen pregnant cows at the third stage were 5.0 ± 1.2 mcg. per g. of tissue.

SUMMARY

The results of the present investigation lead to the following conclusions:

(1) Incubation of progesterone-4-C^{14} with tissue homogenates of normal bovine ovaries led to the formation of testosterone; also $\Delta^{1,4}$-androstadiene-3,17-dione and testosterone were isolated from two cystic ovaries. The percentages of testoserone formed from progesterone in the normal ovaries they were 7.07 and 2.28 per cent, while in the cystic ovaries it was 1.08 and 1.78 per cent. The formation of $\Delta^{1,4}$-androstadiene-3,17-dione from progesterone in one cystic ovary was 0.0142 per cent.

(2) The investigation of the metabolism of non-labeled progesterone in normal and cystic ovaries was also undertaken. When Δ^{4}-androstene-3,17-dione was tentatively identified in the normal ovary, it indicated that testosterone

might be formed by the way of Δ^4-androstene-3,17-dione. Metabolites tentatively identified from the cystic ovaries included Δ^7-allopregnene-3,17-dione and Δ^4-androstene-17β-ol-3,11-dione. From the above information it was postulated that the progesterone might followed a different metabolic pathway in the cystic ovary.

(3) Quantitative analysis of progesterone and its metabolites from bovine corpora lutea, cystic ovarian tissue and follicular fluid were studied by utilizing a micro chemical assay. The average amount of progesterone isolated from corpora lutea at the third stage of pregnancy was 5.2 mcg. per g. of tissue.

(4) Pregnane-3,20-dione was tentatively idenitified from the corpus luteum and androstane-17β-ol was tentatively identified in the cystic follicular fluid.

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THE *IN VITRO* METABOLISM OF C^{14}-LABELED PROGESTERONE IN BOVINE OVARIAN TISSUES

by

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The purpose of this investigation was primarily the study of the _in vitro_ metabolism of progesterone-4-C^{14} in bovine ovarian tissues.

It was undertaken in order to better understand the metabolic role of progesterone in the ovary during the reproductive processes. The study with non-radioactive progesterone was in supplement to the investigation of progesterone or related steroids produced by the bovine corpora lutea, ovarian tissue and follicular fluid. Methods used for the study of the metabolism of C^{14}-labeled progesterone were taken mainly from Samuel's Laboratory at the University of Utah.

Edgar and Gawienowski's methods were used for the qualitative and quantitative analysis of progesterone and related steroids.

From the results of this investigation, the following conculsions were reached:

(1) The cystic bovine ovary can remove the side chain of progesterone-4-C^{14} _in vitro_ and form C^{14}-labeled testosterone and $\Delta^{1,4}$-androstadiene-3,17-dione. The formation of C^{14}-labeled testosterone in the normal bovine ovary was also established. It was postulated that the metabolic pathway of progesterone-4-C^{14} in the cystic ovary was via $\Delta^{1,4}$-androstadiene-3,17-dione, Δ^{4}-androstene-3,17-dione and to testosterone.

(2) Tentatively identified metabolites from cystic bovine ovaries were Δ^{7}-allopregnene-3,20-dione. And Δ^{4}-androstene-17β-ol-3,17-dione. In the normal ovary, Δ^{4}-androstene-3,17-dione was also tentatively identified. It was indicated that progesterone might follow a different metabolic pathway in the cystic ovary than in the normal ovary.

(3) Quantitative analysis of progesterone from bovine corpora lutea of pregnant cows at the third stage of pregnancy revealed an average of 5.2 mcg. of

progesterone per gram of tissue. No correlation was established between the quantity of progesterone isolated and the weight of the tissue.

(4) Hormones tentatively identified from the corpus luteum (sample No. 2) was pregnane-3,20-dione and from the cystic follicular fluid was androstene-17β-ol.